BIOLOGICAL STUDIES ON INTRACELLULAR BACTERIA.[1] NO. 1.

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INTRODUCTION.

It has been known for a long time that certain blattids, homoptera, and ants harbor intracellular non-pathogenic organisms that are transmitted from generation to generation through the egg. Since Huxley's description of the pseudovitellus of aphids in 1858 a host of investigators have studied the embryonic development and morphology of this curious organ and its contents. After Huxley, the most prominent investigators associated with the study of this subject and with the development of the entire field of intracellular organisms in insects, were Lubbock, Metchnikoff, Balbiani, Krassilstschick, Blochmann, Lindner, Heymons, Berlese, Mercier, Pierantoni, Sûlc, Buchner and others.

This vast study has precipitated the following facts and theories which may be presented seriatim.

1. The insects that are definitely known to harbor intracellular non-pathogenic organisms belong to the family Blattidæ, the entire order of Homoptera, and the family Formicidæ.

2. It has also been reported that certain Lepidoptera and Coleoptera harbor intracellular non-pathogenic organisms, but conditions are not clear in the cases cited and the authors may have been confronted with true pathologic cases.

3. In the insects where non-pathogenic intracellular residents have been established one or more definite species of microorganisms are always associated with every individual of a particular species of host.

4. The microörganisms associated with the insects have been called symbionts.

5. The symbionts are transmitted from generation to generation through the egg in a very definite manner.

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6. In adult insects the symbionts are sometimes found free in the lymph, but in most species are found enclosed in very definite, modified cells known as mycetocytes or bacteriocytes.

7. In some species two or more mycetocytes fuse and become concentrated forming a very definite organ known as the mycetome. Some insects are monosymbiotic, that is, they harbor only one species of symbiont; others are disymbiotic or trisymbiotic.

9. When the host is monosymbiotic the symbionts may be found in mycetocytes or may be concentrated in a mycetome.

10. When the host is disymbiotic one species of symbiont may be found in mycetocytes and the other in a mycetome, or both symbionts in separate mycetomes or both symbionts in separate feebly connected mycetomes or lastly both species may be in one mycetome.

11. When the host is trisymbiotic we may have two species of symbionts in one mycetome and the third in mycetocytes or the three symbionts may be in one mycetome.

12. The host cells show specific changes, but no injury. They usually enlarge. If the symbionts are small the cytoplasm has the appearance of being finely reticulate; when large the cytoplasm becomes coarsely reticulate. The nuclei of the host cells are sometimes round; at other times curved or even arborescent. The organisms never invade the nuclei. In spite of deformation of the cytoplasm, the nucleus retains its ability to divide. Sometimes one finds nuclear and cytoplasmic degeneration, but this is an exception and probably due to some secondary parasite or to disease.

13. Most of the mycetocytes are enormous in size when compared with the remaining cells of the animal.

14. The systematic position of the symbionts is not always clear. In the case of the Blattids they are undoubtedly bacteria; in the case of some aphids undoubtedly yeasts, and in the other cases yeast-like organisms and probably higher fungi. No protozoan intracellular symbionts of insects are known.

15. The symbionts from a small number of host species have been cultivated upon artificial media, but in most cases the artificial cultivation experiments of symbionts have so far met with unsurmountable difficulties. This may be due to the fact that

the exact conditions existing in the mycetocytes or mycetome have not been sufficiently reproduced.

16. The number of symbionts per host is enormous. For *Ceroplastes rusci,* Berlese computed the number per host to lie between sixty and seventy thousand.

17. The reasons for the belief that the intracellular organisms are symbionts and not commensuals or parasites are the following:

(*a*) Every individual of a species is infected.

(*b*) The infection produces changes in the host cells, but these are harmless.

(*c*) The infection routes and methods of localization, while different in different hosts and symbionts, follow very definite courses within a species.

(*d*) The microörganisms are numerically controlled by the host, never increasing up to a point where they may prove fatal.

(*e*) The microörganisms within the insects obtain nourishment, and protection from drastic temperature and draught conditions.

18. While numerous theories have ben advanced, the symbiotic relationship, if any exists, has not been established upon any scientific basis.

Buchner (1912) in an extensive paper, together with original morphological investigations, gave an excellent review of all of the literature dealing with the subject of the intracellular symbionts of insects. For this reason, another extensive review of the literature would be merely a duplication of something that has already been admirably accomplished.

In beginning this investigation, I was primarily interested in the physiological aspect of the subject. If the intracellular organisms are really of some use to the host, they may possibly assist in the metabolism of reserve and other foods. This can only be accomplished through the secretion of enzymes by the microörganisms. I therefore thought it well to select one or two species of insects, to attempt to cultivate the intracellular organisms, then to make a systematic study of their enzyme activities, and later to attempt to correlate such activities with the metabolism of the host's diet.

For the above work, I chose two species of cockroaches for the reason that conditions seemed simpler. Cockroaches can be easily obtained and reared. They are large insects when compared with aphids, coccids, psyllids, aleurodids, etc., and can be readily dissected. Moreover, Mercier (1906) reported that he had cultivated the so-called "Blochmann bodies," thus putting an end to the tiresome discussions of Cuenot, Prénant and Henneguy, who supposed that the rods were metabolic products and not bacteria. Mercier cultivated these organisms on nutrient agar, gelatine, potato, milk and bouillon. He described only the morphological characters and named the organism *Bacillus cuenoti*. The fact that one species of cockroach had yielded an organism that could be cultivated also led me to choose these insects in preference to others. Lastly, cockroaches are monosymbiotic; in other words, conditions are not, so far as known, complicated by the presence of more than one microörganism.

Before proceeding to the cultural and physiological section of this paper it might be well to glance over the morphological results in Blattids as shown by Blochmann, Heymons, Buchner and others.

1. The Bacteriocytes and Bacteria in Blattids.

In *Blattela germanica* and *Periplaneta orientalis* certain of the fat cells are filled with bacteria measuring 6 to 8 μ long, straight or somewhat curved. The bacteria divide by binary fission. The cells occupied by the microörganisms do not contain any fat or urates and the nucleus is always normal.

The cockroaches present the bacteriocyte condition; there are no mycetomes nor complexes of mycetocytes nor, more strictly speaking in this case, bacteriocytes.

In *Blattela germanica* several rows of bacteriocytes are found in the fat body. The youngest eggs in the oviduct are free from infection. Somewhat older eggs, however, show several organisms on their surface. With the growth of the egg the number increases so much that later several layers surround the periphery. The direct wandering of the bacteria out of the host cells through the follicular membranes into the eggs, has not been ob-

served, but since the fat body surrounds the ovarioles, this is highly probable.

In *Periplaneta* bacteriocytes have been found in the oviduct. No bacteria have been noticed at the periphery of young oöcytes, but on observing large ones a number can be seen on the surface that have wandered out of the bacteriocytes, and must have made their way out through the follicular membrane. As the egg grows the bacteria become much more plentiful on the surface forming a closed layer around the entire egg. Soon the microorganisms become more concentrated at both poles and many are seen to lie perpendicular to the egg. In freshly laid eggs the bacteria are found within under the vitelline membrane, so they must have left the follicle, but the exact method has not been traced.

In Blattids generally, the later development takes place as follows: The organisms are concentrated in the middle of the yolk. The vitellophags also become infected. When the germ band grows around the yolk the bacteria get into the embryonic intestinal lumen. After a time they leave the lumen, wander through the intestinal epithelium and get into certain fat cells which they modify as previously shown. They take possession of the adipocytes and render them functionless as fat cells.

While the morphological details are not complete, they are very interesting. I found very similar conditions in the two species with which I worked, namely, *Periplaneta americana* and *Parcoblatta virginica.*

One of the most striking things in connection with this work is the definite wanderings of the microörganisms. They are motile forms in the insects studied by me, but that would not account for the prescribed course which they follow. I believe one must postulate a chemotropic response between the bacteria and certain host tissues in order to account for such behavior.

2. The Morphological, Cultural and Biochemical Characters of the Organisms Cultivated from Parcoblatta virginica and Periplaneta americana.

By dissecting cockroaches that portion of the fat body in which the bacteriocytes are embedded can be easily removed. It is

best to sacrifice one or two animals by exploration dissections, and by staining the excised tissue, in order to obtain an idea of the exact position of the desired cells and their microörganisms. When the region of the bacteriocytes has been discovered a fresh animal can be taken, etherized, pinned to a paraffin tray ventral side up, washed off with some sterilizing agent such as alcohol or a mixture of alcohol and corrosive sublimate and dissected with sterile instruments. The excised bacteriocytes with their contents can then be directly placed in the different sterile media and incubated.

The following are descriptions of the morphological, cultural and biochemical characters of the two species of organisms; one from *Parcoblatta virginica* and the other from *Periplaneta americana*. I consider it rather useless to give these bacteria specific names. To begin giving names to all of the thousands of symbionts, I think, would confuse rather than assist matters. It would be far better accurately to designate the host and then simply to refer to the genus of microörganism. In the case of disymbiotic and trisymbiotic insects one can refer to the two or three genera of symbionts associated with the host. If the two or three symbionts all belong to the same genus, the latter can be designated and the organisms labelled *a, b, c,* or 1, 2, 3.

The two species of organisms investigated by me are rather pleomorphic in cultures. Some individuals are straight like a bacillus; others are comma- or crescent-shaped like a spirillum. In the host cells the individuals are nearly all of the spirillum form. For this reason and owing to the fact that the organisms have one polar flagellum and do not form endospores, I am placing them in the genus *Spirillum*.

The organisms found by me in *Parcoblatta* and *Periplaneta* seem to differ from the morphological description of *Bacillus cuenoti* of Mercier. Since the cultural and biochemical characters of *B. cuenoti* were not described it has been rather difficult to make a comparison, however. The organism of Mercier seems to be a true bacillus, forming endospores of oval shape. The two organisms which I cultivated form no endospores.

Spirillum from Parcoblatta virginica.

Morphology.—1.5 per cent. neutral, nutrient agar, short and long rods. In nutrient bouillon longer rods, some curved, others straight. Average length 1.5–2 μ. Stains readily. Gram negative. No endospores. Motile. A single flagellum attached to one of the poles.

Nutrient Agar Stroke.—1.5 per cent. neutral. Growth: abundant, filiform, flat, glistening, smooth, opaque, butyrous. Odor absent. Medium greened.

Nutrient Bouillon.—Ring, clouding strong, odor absent, sediment abundant.

Gelatin Stab.—Growth best at top; line of puncture filiform. Liquefaction begins in two days, crateriform. Color of medium unchanged.

Litmus Milk.—Reaction not changed. Not coagulated. Reduction complete in eight days.

Milk.—Clearing without coagulation. Medium and consistency unchanged. No peptonization.

Potato.—Growth abundant, spreading, flat, glistening, smooth, butyrous. Odor absent. Medium unchanged.

Nutrient Agar Colonies.—1.5 per cent. neutral. Growth rapid at 35° C.; round, smooth, slightly convex, edge entire, amorphous. Diameter 1–1¾ mm.

Nutrient Gelatin Colonies.—Growth rapid, irregular, smooth, crateriform, edge undulate, liquefaction saucer.

Indol test negative.

NH_3 test faintly positive.

Nitrates not reduced to nitrites.

Diastase Test.—Starch converted to sugar.

Strong catalase reaction in bouillon culture.

Faint peroxidase reaction with gum guaiac and H_2O_2 in sugar bouillon culture.

Fermentation of Carbohydrates with Formation of Acid and Gas.

	Gas.	Acid.
Dextrose	0	0
Levulose	0	0
Saccharose	0	0
Maltose	0	0
Lactose	0	0
Mannit	0	0

Spirillum from Periplaneta americana.

Morphology.—1.5 per cent. neutral nutrient agar, short and long rods. Some curved, others straight. In nutrient bouillon, some straight rods and many curved. Average length 1.5–2 μ. Stains readily. Gram negative. No endospores. Motile. A single flagellum attached to one of the poles.

Nutrient Agar Stroke.—1.5 per cent. neutral. Growth abundant, filiform, flat, glistening, smooth, opaque, butyrus, odor absent, medium unchanged.

Nutrient Bouillon.—Ring, pellicle, clouding strong, odor absent, sediment abundant and flocculent.

Gelatin Stab.—Growth best at top, line of puncture filiform, liquefaction begins in two days, crateriform. Medium unchanged.

Litmus Milk.—No acid, coagulation prompt. Prompt reduction.

Milk.—Coagulation prompt, extrusion of whey. Consistency unchanged. Medium slightly greened. No peptonization.

Potato.—Growth scanty, slightly spreading, flat, dull, smooth, butyrous, odor absent, medium unchanged.

Nutrient Agar Colonies.—1.5 per cent. neutral. Growth rapid at 35° C., round, smooth, flat, edge entire, amorphous. Diameter ¾–2 mm.

Nutrient Gelatin Colonies.—Growth slow, colonies minute. Round, smooth, flat, edge entire, liquefaction saucer.

Indol test negative.

NH_3 test positive.

Nitrates not reduced to nitrites.

Diastase Test.—Starch converted to sugar.

Strong catalase reaction in bouillon cultures.

No peroxidase reaction.

Fermentation of Carbohydrates with Formation of Acid and Gas.

	Gas.	Acid.
Dextrose	0	0
Levulose	0	0
Saccharose	0	0
Maltose	0	0
Lactose	0	0
Mannit	0	0

It will be noticed from the foregoing descriptions that the morphological characters of the two species of spirilla are almost identical and if investigation were carried no further, one would doubtless consider the organisms from the two species of cockroaches as identical. Only in the cultural characters do the differences become striking and therefore I believe I am justified in considering the microörganisms as two distinct species. Taking Mercier's meager description of the symbiont from *Blatella* into consideration we should be inclined to assume that every living species of cockroach harbors a separate species of microorganism.

I did not test the pathogenicity of the two cultures on mammals. Perhaps this should have been done, since at present a great amount of interest centers on disease transmission by insects. Cockroaches are known to carry about and disseminate bacteria mechanically on their feet and in their intestines. Nevertheless, investigators interested in this matter might do well also to consider the intracellular organisms. Cockroaches are also in reality living reservoirs of thousands of bacteria enclosed within certain of the hosts' cells.

3. The Biochemical Activities of the Intracellular Organisms Studied.

First of all from the sugar tests it is interesting to note that the intracellular organisms do not ferment any of the sugars used. This is very fortunate for the insects. Undoubtedly sugar is present in some form taken in as food and if fermented by zymase secreted by the symbionts might anesthetize the animals with alcohol and CO_2.

The inverting enzyme sucrase is not produced by the *Parcoblatta* symbiont, but the *Periplaneta* organism inverts saccharose to levulose and dextrose.

Since the organisms are present in the fat body of adults and in the yolk of eggs, it seemed possible that they might have something to do with the metabolism of fat. Experiments with ethyl butyrate, milk fat and other fats failed to demonstrate the existence of lipase.

The cultural features demonstrated the fact that gelatin was

liquefied. In other words it was evident that the organisms secrete a protease. All proteins, however, are not hydrolyzed. For instance, casein does not seem to be effected for after the organisms have been grown in milk for the prescribed time the biuret test did not show the presence of propeptones or peptones.

It was found that both species of intracellular organisms produce diastase. The organisms were grown in sugar-free bouillon for ten days. After that time the cultures were poured into some pure potato starch paste with 2 per cent. thymol and incubated for 48 hours. Thereupon Fehling's solution was reduced showing that sugar had been formed. Check tests accompanied the experimental tests.

The tests for oxidizing enzymes really do not signify much, for they can be obtained with cultures of almost any bacterium such as *B. coli communis* or *B. prodigiosus*. However, both symbionts produce catalase and the *Parcoblatta* organism demonstrated the presence of peroxidase.

We may summarize the enzyme activities on the following table and then attempt to correlate some of them with the metabolism of the insects.

Enzymes Produced by the Intracellular Organisms.

Symbionts.	Zymase.	Invertase.	Diastase.	Protease.	Lipase.	Catalase.	Peroxidase.
Parcoblatta organism	o	o	+	+	o	+	+
Periplaneta organism........	o	+	+	+	o	+	o

It will be seen from the preceding table that the enzymes common to both species of symbionts are diastase, protease and catalase. From the standpoint of Blattid metabolism, I think, diastase and protease important. I was disappointed in not being able to demonstrate the existence of lipase, for conditions generally made me feel sure that there was an association between the microörganisms and fat metabolism.

Both species of insects studied feed primarily on carbohydrates (starches) and proteins, so it was encouraging to be able to demonstrate the two enzymes that effect these two classes of foods. Of course, the insects may feed on fats to some extent, but I think the statement that carbohydrates and proteins constitute their principal diet is fairly safe.

Philiptschenko (1907) studied the physiology of the fat body in *Blattela*. He did not mention the intracellular organisms, but found glycogen, proteins, fats and urates stored in great quantities especially in young animals.

From all of these facts, it is easy to assume that the symbionts assist in the carbohydrate and protein metabolism through enzyme secretion. Of course, the catalase may serve an oxidizing purpose, but I do not attach much weight to such a statement for the reason that protoplasm generally will give this as well as the peroxidase reaction in most cases. Nor do I lay any emphasis on the invertase found in the *Periplaneta* organism. It may serve a purpose, but this is not evident.

4. Conclusions.

In speaking of the intracellular organisms of insects, the word symbiont has been used rather freely in the literature. I have also made use of the word more for the sake of convenience than because I am convinced that we are confronted with true symbiosis. From the morphological studies presented by others, and from my own work it is a great temptation to refrain from thinking of a symbiotic relation. From my experiments it is easy to assume that the insect derives some benefit from the organisms as digestive organs, but what possible benefit can the microorganisms derive? Buchner's suggestions that the intracellular organisms are benefited by being protected within the insects from the drastic atmospheric influences of heat, cold, desiccation, etc., are a little far-fetched. Nor does Buchner's view that the organisms are also assured constant nourishment and opportunity to propagate appeal to me. Millions of bacteria and other organisms not found in the cells of animals manage to live and propagate just as well if not better. It is also necessary to remember that the insect tissue or serum must produce some powerful inhibitor in order to hold the propagation of the microörganisms within a reasonable limit, otherwise the host would be killed. The presence of an inhibitor is surely a handicap in the struggle for existence.

I think the interesting relations discussed had their origin in true parasitism, possibly, in disease. During an early period the

organisms now found as harmless entities within the insects were parasites producing pathologic conditions and disease. It seems to me, that such structures as the mycetocytes, bacteriocytes and mycetomes are a survival of previous profound pathologic changes. Later acquired immunity principles became heritable and the toxins and other effects came under control. The insects could not fully rid themselves of the invaders on account of the fact that transmission from generation to generation had established itself with unerring precision. Still later, the microorganisms lost all of their harmful effects and since they secreted a number of enzymes that proved serviceable to the hosts, the latter were not exterminated, but were probably diverted from their normal phylogenetic course. It is interesting to reflect what would have happened to those insects that harbor intracellular organisms if they had never been invaded by the parasites. If the hosts had not been able to overcome the infection they would have become extinct or at least reduced numerically to an appreciable extent. However, the insects were able to adapt themselves to profound changes and the phylogenetic history must have likewise taken another course. It is impossible to imagine that such structures as the pseudovitellus, mycetocytes and mycetomes with their inhabitants could have developed without altering the physiology and morphology and consequently the habits of the host.

The microörganisms may, as Buchner suggests, derive some benefit from the association, but this is not clear since they probably derived much more benefit as true parasites or disease producers. Judging from the ease with which the Blattid symbionts can be cultivated on almost anything, I feel sure that had they succeeded in exterminating all Blattids, they would still persist today living possibly as saprophytes under much more advantageous conditions.

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